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Lab Practicum Enrichment Projects

- Development of Antibiotic-Resistant Bacteria through Successive Exposure to Streptomycin and Doxycycline
- Investigating antibiotic resistance of lactic acid bacteria in fermented food
- Methodology Development for Extraction and Comparison of Ginsenosides and Ibuprofen

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Development of Antibiotic-Resistant Bacteria through Successive Exposure to Streptomycin and Doxycycline

Gemma Barber and Dominique Tertigas

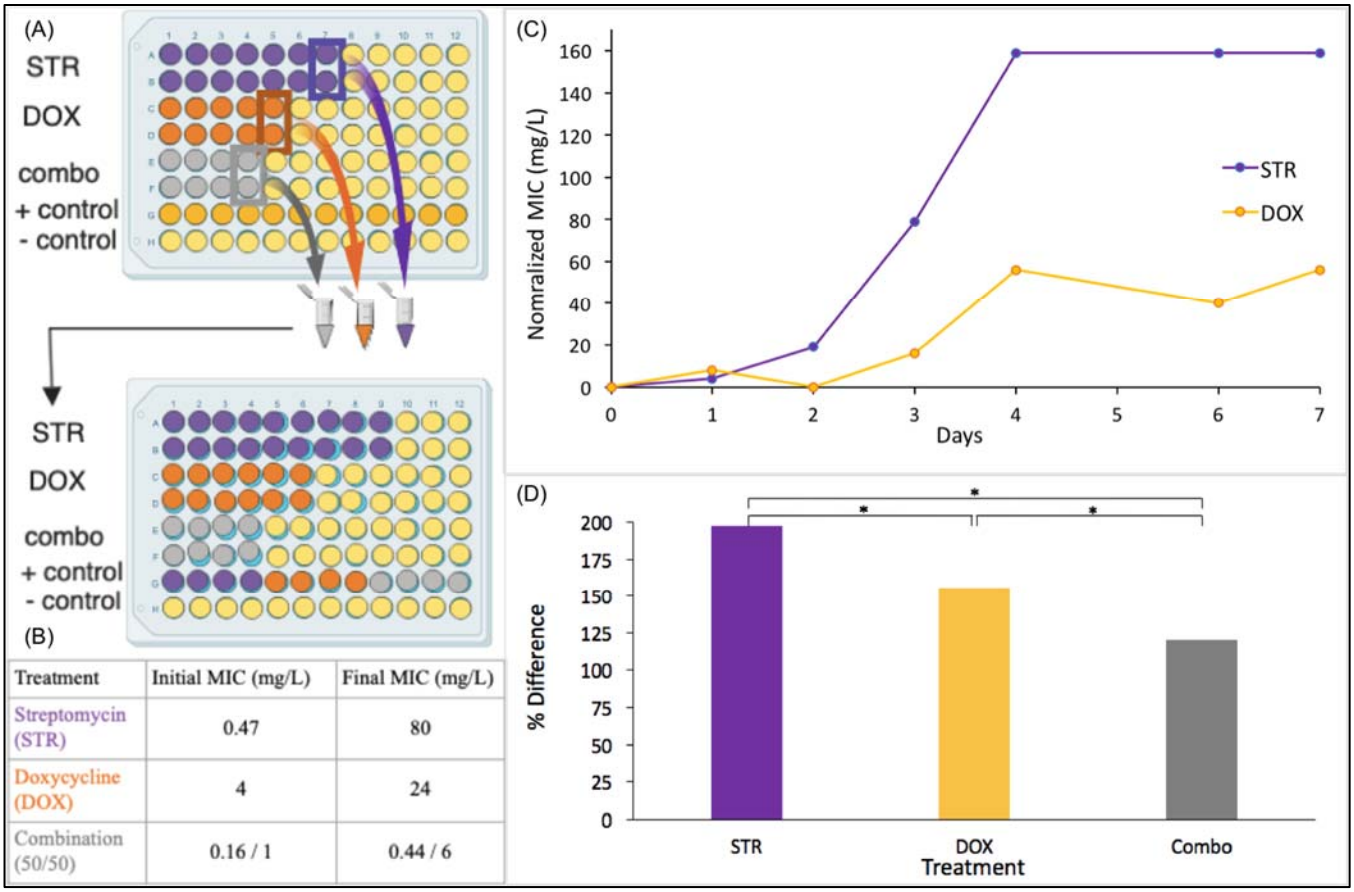
Antibiotic resistance is a pressing issue in the medical field today. Selective pressures leading to antibiotic resistance were investigated by treating wild type *Escherichia coli* with streptomycin, doxycycline and a combination treatment. The minimum inhibitory concentration (MIC) was determined. Bacteria were then isolated from wells with half the concentration of antibiotic as the MIC wells and cultured. This process was repeated over eight generations. The fastest rate of antibiotic resistance was observed for streptomycin. Some cross-resistance was also observed.

Antibiotic resistance is becoming more and more prevalent, resulting in the decrease in effectiveness of many antibiotics[1]. In order to combat this issue, it is important to understand how the rates of antibiotic resistance differ between antibiotics and how this relates to the mechanisms of antibiotic resistance. This can open doors for the implementation of more effective preventative measures in order to minimize the prevalence of antibiotic-resistant bacteria. This study compared antibiotic resistance of two antibiotics in order to better understand how their rates of resistance may differ.

There are several ways by which antibiotic resistance can occur in bacteria. One such mechanism is mutational resistance, where bacteria develop genetic mutations that affect the activity of the drug, thereby leading to predominance of resistant bacteria[2]. These genetic mutations can lead to the decrease in drug uptake in the cell, activation of pumps or efflux mechanisms to remove the drug from the cell, alteration of metabolic pathways or the modification of the antimicrobial target to decrease its affinity for the drug[2]. Furthermore, bacteria can acquire foreign DNA through horizontal gene transfer, which can be accomplished through transformation, transduction or conjugation[2]. Aminoglycosides, such as streptomycin, are known to be affected by aminoglycoside modifying enzymes that modify the hydroxyl or amino groups of the antibiotic[2]. Doxycycline, a tetracycline, may develop resistance through efflux pumps[2]. This study will focus on comparing the rates of resistance of *Escherichia coli* to streptomycin and doxycycline, but future steps would confirm the relationship between the rate of resistance and these underlying mechanisms.

A wild type strain of *Escherichia coli* was selected as the test bacteria due to its susceptibility to both treatments[3]. *E. coli* was treated with streptomycin (STR), doxycycline hyclate (DOX), and a combination of the two antibiotics (both obtained from Sigma-Aldrich). These drugs were chosen based on results of a study by Oz et al. (2014), which showed that their rates of resistance were different[3].

Minimum inhibitory concentration (MIC) values were determined in 96-well plates with each antibiotic added in duplicate (Figure 1 (A), (B)). Difco™ Nutrient Broth was added to all wells: 50 µL in rows A-G and 100 µL in row H. Row H acted as a negative control, as it contained only nutrient broth. 50 µL of STR at a concentration of 80 mg/L was added to well 12 of rows A and B. This concentration was changed to 5120 mg/L as the *E. coli* continued to grow at higher concentrations. 50 µL of DOX at a concentration of 512 mg/L was added to well 12 of rows C and



Treatment	Initial MIC (mg/L)	Final MIC (mg/L)
Streptomycin (STR)	0.47	80
Doxycycline (DOX)	4	24
Combination (50/50)	0.16 / 1	0.44 / 6

Fig. 1 | Methods, results and analysis of rate of resistance study. (A) Schematic diagram of the process of isolating bacteria from MIC/2 wells which were then grown and added to a new 96-well plate. The respective treatment colours in this diagram refer to growth. This process was repeated for eight generations. Figure created using BioRender. (B) Recorded MIC for the first and last generations of the three treatments. (C) Normalized MIC of STR and DOX treatments over eight generations. (D) Percent difference of final and initial MIC of all treatments (n = 2) over eight generations (* = $P < 0.05$).

D. For the combination treatment, the highest concentration consisted of 40 mg/L of STR and 256 mg/L of DOX. For this treatment, 25 µL of the specified STR concentration and 25 µL of the specified DOX concentration were added into row 12 of E and F. Two-fold serial dilutions were performed from well 12 to well 1 of rows A to E. For the initial well set-up, 50 µL of wild type *E. coli* with an optical density at 610 nm between 0.08 and 0.10 was added to all wells in rows A to G. Row G acted as a positive control, as it contained 50 µL nutrient broth and 50 µL wild type *E. coli*.

After all components were added, the plate was incubated at 37˚C for 24 hours, at which point it was read using a light and a mirror to determine the MIC of each antibiotic. A scale was created from zero to four, where zero referred to no growth and four referred to maximum growth seen in the positive control well. The MIC was defined as the first well where no bacterial growth was observed. Bacteria were isolated from the MIC/2 wells, or the last wells with bacterial growth. Bacteria isolated from the same treatment were combined and centrifuged, the supernatant was removed, and the pellet was resuspended in Difco™ Nutrient Broth and grown overnight in a water bath at 37˚C. These bacteria were then used to repeat the plating procedure outlined above. Bacteria were added to the same treatment from which they had been isolated. For example, bacteria isolated from the wells treated with STR were used for the next generation of STR treatment. Isolated bacteria from each treatment were also added to four wells in the positive

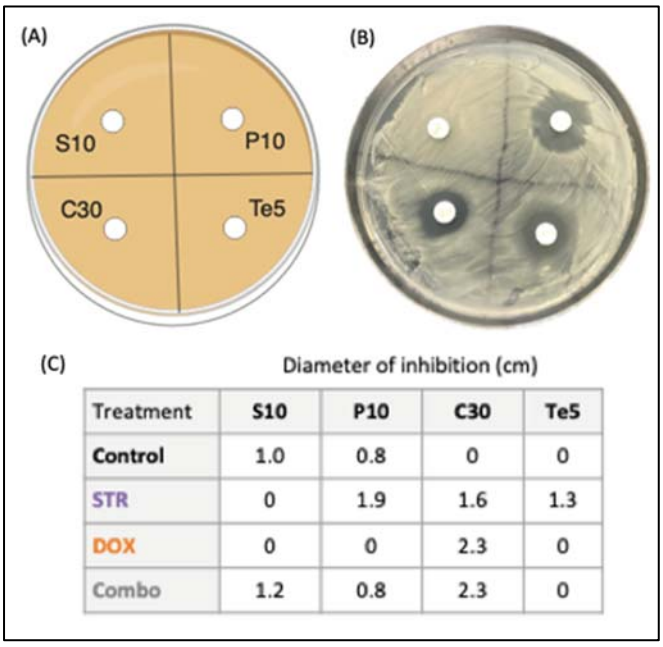
control row. This procedure was repeated for eight generations.

Percent difference comparing the final generation MIC and initial generation MIC for both replicates of each treatment was determined. This allowed for the comparison of the change in MIC over the experiment between treatments. An ANOVA was performed in order to test whether at least one pair of treatments were statistically different, which was followed by a post hoc Tukey test. Despite a low power due to the sample size, this analysis provides a methodological basis for future studies with larger sample sizes.

Bacteria treated with STR was observed to become resistant at a faster rate than the bacteria treated with DOX (Figure 1 (C)). This can be seen by comparing the change in MIC of each generation as compared to the initial MIC. Similar trends were seen in a paper by Oz et al. (2014)[3]. This is possibly due to a difference in mechanism or amplification of resistance between these two antibiotics[4].

When analyzing the percent difference (comparing the final and first generations' MIC) for each treatment, the change in MIC was statistically different ($P<0.05$) between STR and DOX, STR and combination, and DOX and combination (Figure 1 (D)). This further confirms that STR became resistant at a faster rate than DOX. It also shows that the combination treatment became resistant at an overall slower rate compared to STR and DOX. A possible explanation is that it may be more difficult to become resistant to two different antibiotics at the same time.

While the rate of resistance is important to consider, breakpoint values are also essential. Breakpoints are universal values defined as the concentration at which bacteria are resistant to an antibiotic based on their MIC[5]. For STR, this is 32 mg/L [6], which was surpassed by day 3. For DOX, a tetracycline, it is 16 mg/L [6], which was reached on day 4. The combination treatment never surpassed either of these breakpoint values. This supports the notion that a combination treatment of these two antibiotics will lead to a slower rate of resistance than treatment with a single antibiotic.



Treatment	S10	P10	C30	Te5
Control	1.0	0.8	0	0
STR	0	1.9	1.6	1.3
DOX	0	0	2.3	0
Combo	1.2	0.8	2.3	0

Fig. 2 | Set-up and results of cross-resistance study. (A) Schematic diagram of the set-up of the cross-resistance test plate with four different antibiotic disks. Four of these plates were prepared, with each being for a different treatment. (B) Image of STR-treated bacteria plate. No ring of inhibition is seen around the S10 antibiotic disk, but all other antibiotics had an effect. (C) Results are shown in a table with diameter of inhibition being measured as the diameter of the area around the antibiotic disk where no bacterial growth was observed. A higher diameter of inhibition indicates a higher efficacy of the antibiotic or a lower level of resistance of the bacteria.

Cross-resistance was tested by plating 100 µL of each bacteria treatment and the control (initial wild type *E. coli*) on four different petri dishes containing Difco™ Nutrient Broth agar. Each plate was split into quadrants, and an antibiotic disk was placed in each quadrant. The disks used were 10 µg streptomycin (S10), 10 µg penicillin (P10), 30 µg chloramphenicol (C30) and 5 µg tetracycline (Te5). These plates were incubated for 24 hours at 37˚C and diameter of inhibition was measured.

The resistance of these bacteria was further confirmed in this test as STR-treated bacteria were resistant to

the S10 antibiotic disk and DOX-treated bacteria were resistant to the Te5 antibiotic disk. This test also showed some incidence of cross-resistance; DOX-treated bacteria became resistant to streptomycin and penicillin. This can be seen by comparing the diameter of inhibition of the control to the diameter of inhibition of DOX-treated bacteria (Figure 2). Bacteria from all treatments became more susceptible to C30 as compared to the control. There was no inhibition by this antibiotic disk on the control, but all other treatments were inhibited to some degree by C30 (Figure 2). P10 and Te5 were more effective against STR-treated bacteria than the control. The combination treatment exhibited almost no changes from the control, indicating that very little resistance had been implemented in these bacteria. These results can also be explained by the differences in mechanism and/or amplitude of resistance between each treatment[2].

There are many implications of this study that can be applied to a clinical setting. This study showed that combination treatments reduce the antibiotic resistant bacteria. Research comparing monotherapies and combination antibiotic treatments currently remain inconclusive and controversial[7]. The question that remains to be answered is what the underlying cause of the difference in resistance rates is. Understanding the mechanisms of resistance and how this relates to the structure and mechanism of each antibiotic can be achieved through whole genome sequencing[8,9]. Knowing which structures and classes of antibiotics lead to slower rates of resistance in bacteria could allow for the creation of new antibiotics that would induce slower rates of resistance.

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Investigating antibiotic resistance of lactic acid bacteria in fermented food

Meea Fogal and Brittany Mascarenhas

Antibiotic resistance is a prevalent issue affecting both the environment and public health. In more recent years, bacteria used in food production, such as lactic acid bacteria (LAB) from dairy products, have been shown to be large contributors to this crisis. To assess this topic, we performed a multi-step assay involving isolating LAB from yogurt samples, culturing and characterizing the bacteria, and assessing their resistance to common antibiotics.

Antibiotic resistance arises when a microorganism acquires the ability to evade the inhibitory effects of antimicrobial agents[1]. This often occurs in bacterial species possessing conjugative determinants allowing for the transmission of resistance-inducing genes[2]. One such order of bacteria is lactic acid bacteria (LAB)[1]. LAB are able to form lactic acid and other metabolic outputs such as hydrogen peroxide and bacteriocins as byproducts of fermentation. Due to these products, LAB are commonly utilized in commercial foods and animal feed as starter cultures and shelf life extenders[1]. This, however, has implications, as these applications force LAB into the food chain, which is a prominent source of resistant bacteria transmission, further exacerbating the ongoing crisis of bacterial resistance. As a result, rigorous testing before applications in both humans and animals is vital[3].

In this study, we aimed to investigate the presence of antibiotic resistant LAB in yogurt, a commonly consumed fermented food product. We hypothesized that through our multi-step assay (Fig. 1) we would be able to effectively isolate and identify LAB in yogurt and assess the efficacy of several different antibiotics on their inhibition.

To facilitate the isolation of single colonies, 22 mL-De Man, Rogosa and Sharpe (MRS) agar plates were created by dissolving 50 mg MRS agar powder (Weber Scientific) in 1 L of distilled water (dH_2O) and autoclaving at 121°C for 30 minutes. Before pouring the plates, 50 mg of pure nystatin dissolved in 1 mL of sterile water was added to the agar to prevent unwanted growth of fungi, which may occur through contamination.

Single colonies of bacteria were first isolated from Organic Meadow® 2% probiotic yogurt containing active bacteria cultures of *Lactobacillus acidophilus*, *Bifidobacterium* spp., *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. The yogurt sample was initially diluted 1:2 using sterile water (sterile nutrient broth diluent produced similar results). Five 1:2 serial dilutions were conducted, and 0.5 µL of each of the 1:4, 1:8, 1:16, 1:32, and 1:64 dilutions were plated on MRS agar. Plates were incubated at 35°C for 72 hours.

To characterize and identify bacterial isolates, single bacterial colonies were transferred to liquid medium. Using a wire loop, colonies with distinct morphologies (big smooth (BS), small smooth (SS), big rough (BR), and small rough (SR)) were selected and used to inoculate four separate 15 mL centrifuge tubes containing 5 mL of Lysogeny broth (LB); (nutrient broth did not produce adequate growth). Liquid cultures were loosely capped to allow for oxygenation and incubated at 37 °C in a water bath for three days. All four liquid cultures were monitored for growth by comparison to appearance of sterile LB media. It was determined that both rough colonies (BR and SR) did not grow in liquid culture, while both smooth colonies (BS and SS) showed an increase in

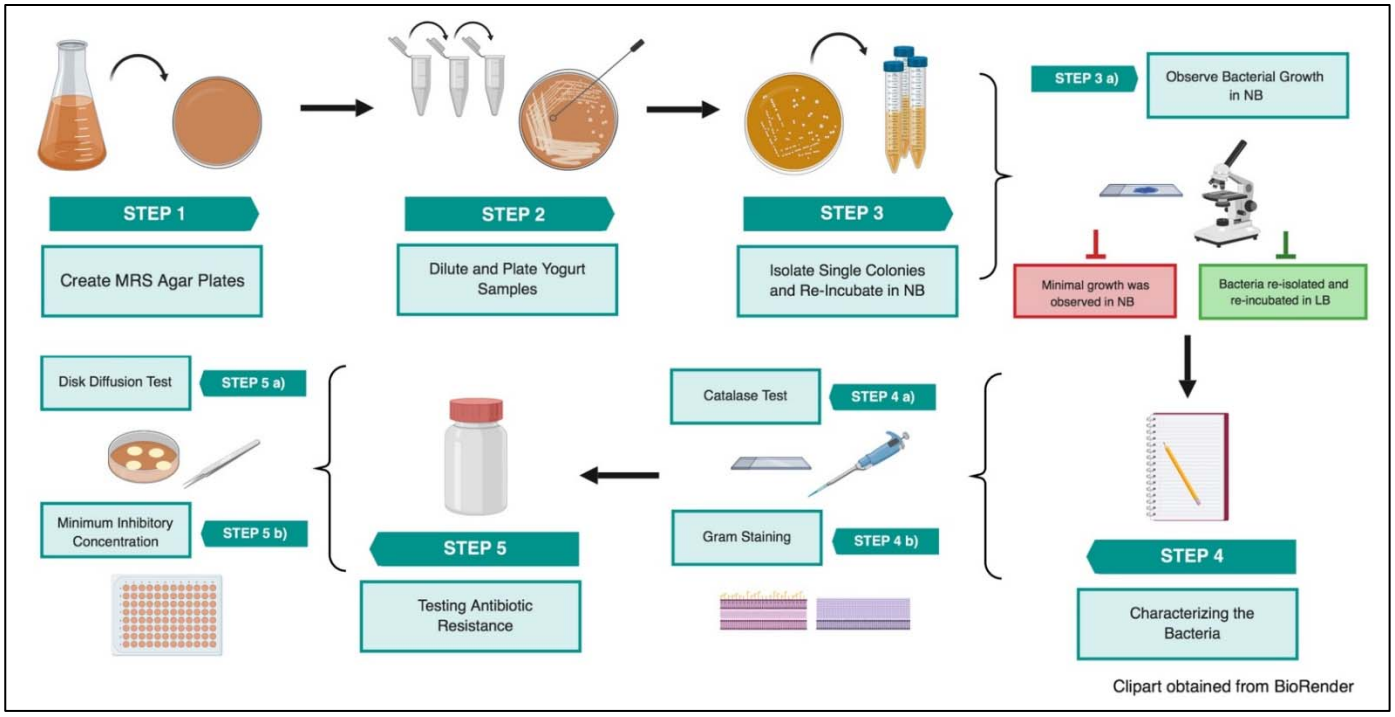


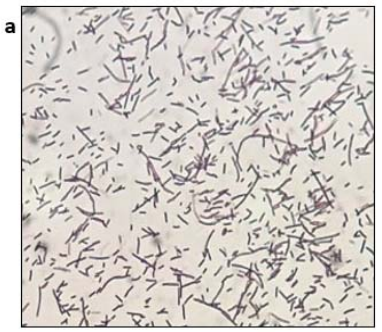
Fig. 1 | Flow chart outlining the multi-step assay for bacterial isolation and testing. Step 1 involved creating solidified MRS agar plates from powder. In Step 2 serial dilutions on yogurt samples were performed and the resulting concentrations were plated on the MRS agar plates. In Step 3, single colonies grown on the agar plates were isolated and re-incubated in nutrient broth (NB) which resulted in minimal observable growth. Bacteria was re-isolated from the plates and suspended in Lysogeny broth (LB) which provided greater growth. Step 4 involved the characterization of isolated bacteria, through a catalase test (a) and gram staining (b). Testing antibiotic resistance occurred in Step 5, through use of a Disk Diffusion test (a) and Minimum Inhibitory Concentration test (b).

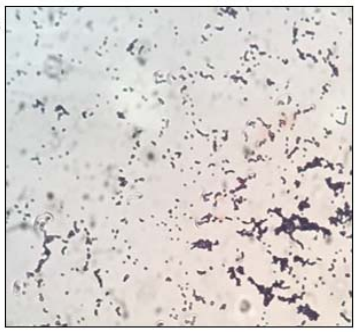
turbidity, indicative of growth. This observation was validated by re-plating all liquid bacterial cultures and observing no growth for rough colonies but growth for smooth isolated bacterial colonies.

To further characterize all isolates, Gram staining was performed. The reagents, which included crystal violet, iodine, 2.5% safranin, and 95% ethanol, were made fresh. The procedures for preparation of slide smears (2 for each type of bacteria) and gram staining were followed from a protocol outlined by the World Health Organization, 2003[4]. All slides were assessed under a light microscope after staining was completed. Both BS and SS colonies were shown to be Gram positive cocci, which is typical of LAB. BR and SR were Gram positive rod shaped bacilli, indicating that these isolates were distinct from the smooth colonies (Fig. 2 a,b).

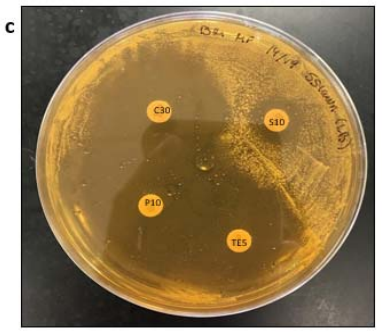
For additional characterization, a catalase test was performed. Using a sterile wire inoculation loop, a colony of SS bacteria from the initial plates was evenly spread into a circle approximately 1 cm in diameter on a glass microscope slide. A drop of 3% w/v hydrogen peroxide (Exact™) was then distributed on the smear of bacteria using a transfer pipette. The sample was observed for a few seconds for the evolution of bubbles. This process was repeated for the remaining bacterial types, BS, SR, and BR, as well as for a known strain of *Lactobacillus plantarum*, acting as a control. From the catalase test it was determined that all isolated bacteria were catalase negative, which is a characteristic feature of LAB.

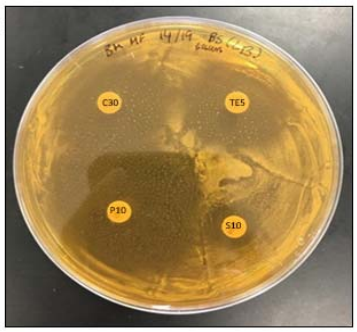
Lastly, to assess antibiotic resistance, a disk diffusion test and Minimum Inhibitory Concentration test (MIC) were performed. The disk diffusion test was





Colony	Morphology	Gram stain
BS	Cocci	Gram positive
SS	Cocci	Gram positive
BR	Bacilli	Gram positive
SR	Bacilli	Gram positive





Colony	Antibiotic	Mean Diameter (cm)
B.S	Chloramphenicol (30µg)	3.7
	Tetracycline (5µg)	3.3
	Streptomycin (10µg)	1.5
	Penicillin (10µg)	4.7
S.S	Chloramphenicol (30µg)	4.1
	Tetracycline (5µg)	3.25
	Streptomycin (10µg)	1.6
	Penicillin (10µg)	5.0

Fig. 2 | Characterization of bacterial isolates and efficacy of four antibiotics. a, Gram stain results of rough colonies, determined to be rod-shaped and Gram positive. **b**, Gram stain results of smooth colonies, determined to be cocci and Gram positive. Gram stain results of each colony are shown on table to the right. **c,** BS and **d,** SS disk diffusion test diameters of four antibiotics: chloramphenicol (C), tetracycline (TE), penicillin (P), streptomycin (S). Bacteria was determined to be most resistant to streptomycin in both BS and SS colonies with diameters of 1.5 cm and 1.55 cm, respectively. Inhibition zone values for each antibiotic are shown on a table to the right.

performed in duplicate on only smooth colonies (BS and SS), as rough colonies did not grow in liquid culture. A P200 micropipette (Biohit) was first used to apply 100 µL of the BS liquid culture onto the middle of two MRS agar plates, followed by even surface spreading with a cell spreader. These steps were repeated for the SS liquid culture. Lightly flamed forceps were used to place 4 antibiotics discs (streptomycin (S10), chloramphenicol (C30), penicillin (P10), and tetracycline (TE5)) (Becton, Dickinson & Co) equidistant on each plate. Plates were incubated at 37 °C for three days. The disk diffusion test revealed similar efficacy of each antibiotic for BS and SS colonies (Fig 2 c, d). This, in addition to highly similar Gram stain and catalase test results for both BS and SS colonies, validated our use of solely BS colonies for subsequent testing.

The MIC test involved using the antibiotics streptomycin (STR) and doxycycline (DOX) and a positive and negative control. The initial stock concentration of STR was made by dissolving 53.4 mg of STR powder (Sigma Aldrich) in 0.130 mL dH_2O for an initial concentration of 409600 µg/mL. The initial stock concentration of DOX was made by dissolving 10.7 mg of DOX power (Sigma Aldrich) in 0.0327 mL dH_2O for an initial concentration of approximately 327 680 µg/mL. Serial dilutions were performed to yield solutions of STR and DOX at 10240 µg/mL and 8192 µg/mL, respectively, which were the starting concentrations used in the 96 well plate. All wells were filled to final volumes of 100 µL with varying volumes of LB, starting drug concentrations, and bacterial suspension. Once complete, the plate was covered and incubated at 37 °C and assessed after both 24 hours and 48 hours due to slow bacterial growth.

As pertaining to the goals of this study, the results of our characterization methods supported the isolation of LAB, and we were able examine the efficacy of

antibiotics in inhibiting growth of our bacterial isolates from the yogurt sample. It was determined that the least effective antibiotic was streptomycin, as this drug produced the smallest inhibition zone in the susceptibility test on average when compared to all other antibiotics. Upon further examination through an MIC test, it was determined that streptomycin had an MIC of 2560 µg/mL while doxycycline had an MIC of 512 µg/mL.

Streptomycin is an aminoglycoside antibiotic with selective activity against both Gram positive and Gram negative bacteria[5]. Given that the bacteria isolated from the yogurt sample was determined to be Gram positive, streptomycin should theoretically inhibit growth of LAB via the inhibition of polypeptide synthesis by binding to 16S rRNA[6]. Tetracycline antibiotics have a similar mechanism of action to streptomycin but instead act on the 30S ribosome[7]. This explains susceptibility of the LAB to tetracycline.

Penicillin acts mainly on Gram positive bacteria and chloramphenicol is a broad-spectrum antibiotic acting on both Gram positive and Gram negative bacteria, explaining the isolated LAB's susceptibility to both antibiotics[8,9]. The results of the antibiotic susceptibility test indicate the presence of inherent rather than acquired resistance to streptomycin, as the bacterial structure did not prove to be a limiting factor to the mechanism of action of the antibiotics.

On further testing of the LAB isolate, it was found that the aminoglycoside antibiotic streptomycin had a higher MIC (2560 µg/mL) than the tetracycline antibiotic doxycycline (512 µg/mL). This may be indicative of a degree of resistance in the isolated LAB, as when compared to another broad-spectrum antibiotic with a similar mechanism (DOX), a higher dosage of streptomycin is required to inhibit bacterial growth. However, these results may be due to differences in binding sites of each antibiotic. To further assess antibiotic resistance and determine the genus and species of isolated bacteria, PCR could be used to sequence 16S rRNA to detect antibiotic resistance genes, and for comparison to sequences of known species to identify the isolated bacteria[10].

From this study it is evident that antibiotic resistant LAB may be present within fermented foods, which may potentiate the role of the food chain in the transmission of bacterial resistance. With this, the presence of antibiotic resistant LAB in fermented food products should further be assessed in their role as contributors to the crisis of antibiotic resistance.

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Methodology Development for Extraction and Comparison of Ginsenosides and Ibuprofen

Kate Kim and Jasmine Yang

Natural remedies are widely discredited. Methods to extract the active ingredients of ginseng and Advil were developed for the purpose of identifying similarities, potentially leading to their shared medicinal effect. Extraction success of ginsenosides, the active ingredient of ginseng, was confirmed using liquid chromatography tandem-mass spectrometry (LC-MS/MS). LC-MS/MS data for ginsenosides and ibuprofen, the active ingredient of Advil, were compared. No similarity in chemical composition was found.

For centuries, medicinal plants have been used as natural remedies to provide relief to ailments, similar to that provided by current Western medicine. In some cases, certain Western medicines cannot be taken due to circumstances such as pregnancy, allergic reactions, or side-reactions with other medicine the individual intakes [1]. Searching for scientific validation of natural remedies with a rich history of their use is important since it can offer an alternative for these individuals.

The scope of this project is to develop a method for extracting the active ingredients of the medicinal plant, ginseng, and its Western counterpart, Advil. Both medicines are used to reduce inflammation and the extraction of their active ingredients, ginsenosides and ibuprofen respectively, can allow for further chemical analysis to determine the mechanisms leading to ginseng's observed ability to reduce inflammation [2-4]. Ginseng and Advil were chosen out of accessibility, as both require no prescription.

Methodology

A comprehensive review of extracting natural products by Zhang et al [5]. suggested using decoction for the extraction of ginsenosides. Decoction involves boiling the sample to concentrate on the natural product. Reflux extraction is a type of decoction where the dissolved sample is heated in a flask attached to a cooled column, and the condensate formed on the walls of the cooled column is collected and analyzed. It was chosen as the preliminary method of extraction for this study, which was supported by the findings of Corbit et al., who performed sonication, water, and reflux extractions to determine the optimal method for the extraction of ginsenosides from the ginseng roots [6]. They found that reflux extraction obtained the highest number of ginsenosides and produced the highest yield of each extracted ginsenoside.

Over 60 types of ginsenosides have been identified in ginseng root. Qi et al. identified the major types of ginsenoside present as protopanaxadiol (PPD) and protopanaxatriol (PPT) [7]. PPDs have a base chemical composition of $C_{27}H_{46}O_2$ and contain three variant groups. PPTs have a base chemical composition of $C_{27}H_{46}O_3$ with three variant groups as well. Major ginsenosides identified were Rb1, Rb2, Re, Rg1, and Rc [6,7]. Thus, these were the target ginsenosides for the extraction in this study. Corbit et al. used 100% methanol for reflux extraction, but since the variant groups would lead to different chemical properties, a more universal solvent, 100% ethanol was used [6]. Furthermore, ibuprofen is soluble in pure ethanol, so the same solvent and procedure of reflux extraction can be used on both ginseng and Advil samples, increasing consistency and the magnitude of control applied to the experiments [8].

Sample Preparation

Pure Canadian ginseng root in dried form was obtained from a local grocery store. To increase dissolution of ginsenosides into the solvent, the ginseng root was wrapped

with a paper towel and broken into small pieces using a hammer. The broken pieces were then ground up using a mortar and pestle to a powder form, which was sealed in a container and stored at 4°C [9].

Regular strength Advil was obtained from a local drug store. It was ground up using only mortar and pestle to a powder form. The powder was sealed in a container and stored at room temperature.

Reflux Extraction of Ibuprofen and Ginsenosides from Advil and Ginseng

The standard method of reflux extraction was used to extract ibuprofen and ginsenosides from Advil and ginseng [10]. Either 0.85g of Advil or 2.0g of ginseng powder was added to the reflux vial with 7 mL of 70% ethanol in two separate experiments. The condensates collected in each experiment were used for thin-layer chromatography (TLC) visualization.

Dissolution Extraction of Ibuprofen from Advil

In a 15mL polypropylene tube, approximately 0.2g of Advil powder was added to 2mL of 100% ethanol. The tube was left on an automatic rocking machine at room temperature for 24 hours, after which the liquid layer was separated using a plastic pipette and TLC visualization was performed to confirm the extraction.

Dissolution Extraction of Ginsenosides from Ginseng

In two 15mL polypropylene tube, approximately 0.1g of ginseng powder was combined with 4mL of isopropanol for each tube. The tubes were left on an automatic rocking machine at room temperature for 24 hours, after which the liquid layer was filtered using a vacuum filtration method. The filtrate was then condensed using a rotary evaporator and stored at 4°C [9, 10]. TLC visualization was performed followed by LC-MS/MS to confirm extraction success.

TLC Visualization

For the reflux extraction, a sample of the ginseng condensate, Advil condensate, and a salicylic acid control was dotted onto the silica gel strip using capillary tubes. The control was created by dissolving roughly 0.1g of salicylic acid in 1mL of methanol. The TLC strip was then left in a TLC jar containing 30mL of acetone-petroleum ether (1:4) [11]. Upon completion, 285nm ultraviolet (UV) light was used to visualize salicylic acid and ibuprofen since both compounds contain a benzene ring and would fluoresce [12]. Ginsenosides do not contain aromatic rings, and thus would not be visible. No spots were observed for ibuprofen under 285nm UV light.

In the dissolution experiment, the TLC strip was prepared and ran in the same manner as the reflux extraction trial. UV light visualization showed the presence of salicylic acid and ibuprofen. For the TLC strip with ginsenoside sample, it was dipped in methanol-sulfuric acid (9:1) for three seconds and heated using a hairdryer. This process is known as the Anisaldehyde-sulfuric acid stain and visualizes sugars, which ginsenosides all contain [7,13]. Browning appeared on the strip, indicating the successful extraction for ginsenosides [7,13].

LC-MS/MS Data Acquisition for Ginsenosides

LC-MS/MS was used only on the ginseng sample and not ibuprofen due to budget limits. 1mg of the ginseng sample was reconstituted in acetyl nitrile and diluted 20-fold. The solution was placed into the Agilent 1200 HPLC machine and was run using 10% formic acid in water as the eluting buffer and 10% formic acid in acetyl nitrile as the standard buffer. The Phenomenex Luna 3um C18(2) 150 x 2.0mm 3µm 100A column was used and the sample ran for 18 minutes at a flow rate of 0.2mL/min. MS/MS data were collected using the Agilent 6550 QTOF machine and interpreted using Agilent MassHunter Qualitative Analysis B.7.0.00 software.

LC-MS/MS Data Acquisition for Ibuprofen

LC-MS/MS data for ibuprofen was obtained from the Metlin database [14].

Results

The reflux extraction did not show any results for TLC visualization. While the control with salicylic acid was present under 285nm UV light, ibuprofen and ginsenosides did not

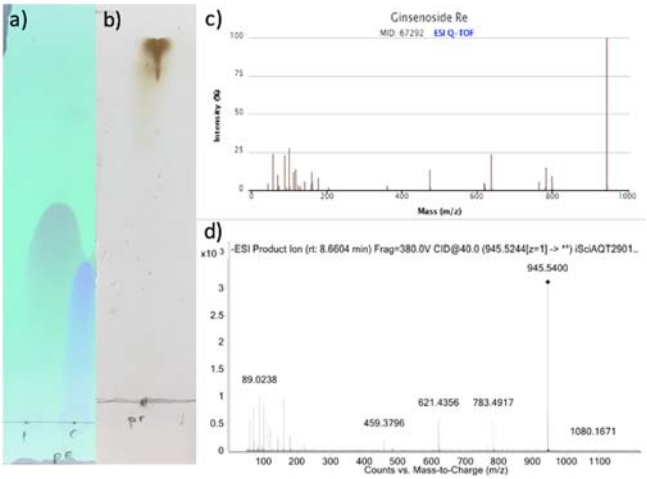

Fig. 1 | Proof for extraction of ibuprofen and ginsenosides using TLC and LC-MS/MS. a. TLC visualization for ibuprofen extraction using acetone-petroleum ether (1:4) under 285 nm UV light. Benzene fluorescence indicating the presence of ibuprofen shown on the left side of the plate, with salicylic acid (control) on the right. **b.** TLC visualization for ginsenosides extraction using acetone-petroleum ether (1:4) with anisaldehyde-sulfuric acid staining method. The brown staining shows the existence of sugars, indicating the presence of ginsenosides. **c.** LC-MS/MS data for ginsenoside Re obtained from the Metlin database. **d.** LC-MS/MS data obtained for the ginsenoside Re in the sample prepared. Comparison between c and d shows similar peaks, confirming successful extraction [14].

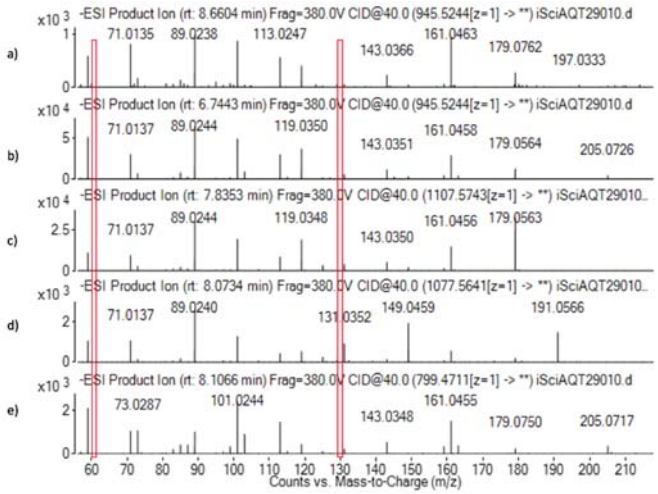

Fig. 2 | LC-MS/MS data for extracted ginsenosides compared with LC-MS/MS data for ibuprofen obtained from Metlin database. a. LC-MS/MS data for extracted ginsenoside Re. **b.** LC-MS/MS data for extracted ginsenoside Re, detected by mass spectrometer at the later time point than a. **c.** LC-MS/MS data for extracted ginsenoside Rc and Rb2. **d.** LC-MS/MS data for extracted ginsenoside Rb1. **e.** LC-MS/MS data for extracted ginsenoside Rg1. The overlaid red bars indicate locations where ibuprofen peaks should be (at 61.0356 m/z and 130.0125 m/z). Comparison between the LC-MS/MS data for ginsenosides and ibuprofen showed no similar peaks [14].

show. This is expected for ginsenosides, as 285nm UV light only visualizes compounds with benzene rings, and only salicylic acid and ibuprofen are aromatic [12]. Ibuprofen most likely did not show due to a low concentration in the condensate. Looking at the chemical structure of ibuprofen, it has low volatility due to its large size and strong intramolecular forces [3]. Thus, most of the compound likely remained in solution rather than being evaporated and collected in the cooled column. Dissolution was used instead to circumvent the volatility problem. UV light visualization indicated successful extraction for ibuprofen using dissolution in pure ethanol. Next, an anisaldehyde-sulfuric acid stain was performed on the ginseng sample dissolved in pure isopropanol and condensed using rotary evaporator. The sugars in the ginsenoside reacted with the sulfuric acid and browning appeared on the TLC strips, indicating successful extraction (see Figure 1). LC-MS/MS was then performed and data confirmed the successful extraction of ginsenosides Rb1, Rb2, Re, Rg1, and Rc specifically.

Next, LC-MS/MS data for ginsenosides was compared to that of ibuprofen. While ibuprofen has peaks at 61.0356m/z and 130.0125m/z, the extracted ginsenosides did not show similar peaks - see Figure 2 [14]. Therefore, there are no similarities between the chemical composition of ibuprofen and ginsenosides. Since the peaks observed are based on the fragmentation pattern, which depends on interactions within the molecule, the data obtained simply shows that ginsenoside and ibuprofen compounds have different intramolecular forces [15]. Further analysis of both compounds needs to be performed to understand why ginseng has shown to work in individuals, as LC-MS/MS cannot indicate the difference in interactions

between ginseng and Advil and the human body. Thus, using the extraction method developed in this project, further analysis should be performed to provide scientific substantiation for the use of ginseng as the natural remedy of Advil.

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